Koisio Technology-Produced Water Significantly Increased the Abundance of Beneficial Gut Bacterium and Decreased the Abundance of Harmful Gut Bacterium of Mice

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Running Title: *Koisio water improves gut microbiota*

Abstract

Increasing evidence has indicated that gut microbiota plays crucial roles in multiple important biological processes such as energy metabolism and immunological functions. Alterations of gut microbiota also contribute significantly to the pathogenesis of a number of diseases. Our previous study has reported that Koisio technology-produced water (KW) led to significantly decreased inflammation and oxidative stress in both cell culture studies and animal studies. In this study we investigated the effects of KW drinking on the gut microbiota of mice, obtaining the following findings: First, KW drinking significantly increased the abundance of several beneficial genera of the gut bacterium including *Akkermansia*, *Faecalibaculum*, *Ligilactobacillus*, *Lachnospiraceae* and *Roseburia*; second, KW drinking significantly decreased the abundance of several harmful genera of the gut bacterium including *Clostridioides, Escherichia-Shigella*, and *Enterococcus*; and third, KW drinking significantly increased the abundance of Verrocomicrobiota, while it significantly decreased the abundance of Proteobacteria of the gut microbiota. Moreover, drinking of KW significantly increased the diversity and richness of the gut microbiota. Collectively, our study has obtained novel findings that KW is capable of not only increasing significantly the abundance of beneficial gut bacterium and decreasing significantly the abundance of harmful gut bacterium, but also increasing the diversity and richness of the gut microbiota of mice.

Keywords: Beneficial gut bacterium; Harmful gut bacterium; Abundance; Koisio water; Mice.

Introduction

A large number of studies have indicated that alterations of gut microbiota is novel common mechanisms underlying a number of diseases, novel common biomarkers for numerous diseases, and novel common therapeutic targets for numerous diseases [1-6]. The gut microbiota plays important roles in multiple biological processes such as nutrient digestion, host immunity, and defense against pathogenic microbial colonization [3, 6, 7]. Numerous studies have also indicated that alterations of gut microbiota are causative to multiple diseases, including metabolic diseases such as Type II diabetes, diseases of digestive system such as inflammatory bowel disease, neuropsychiatric diseases such as autism, and age-associated diseases [2-5]. Therefore, it is of significant scientific and medical significance to find novel approaches that can enhance the healthy state of gut microbiota.

Our previous study has suggested that Koisio technology-produced water (KW) has significant antioxidant capacity [8] and anti-inflammatory capacity [9]. Since inflammation and oxidative stress may produce significant effects on gut microbiota [10], we hypothesized that KW drinking may lead to changes of gut microbiota. In this study we used a mouse model to test this hypothesis. Our study has found that KW produced significant beneficial effects on gut microbiota, which can increase significantly the abundance of beneficial gut bacterium and decrease significantly the abundance of harmful gut bacterium of mice.

Materials and Methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except where noted. KW was produced by the technical experts of Shanghai Koisio Food Industry Co. (Shanghai, China) according to standard procedures.

Methods

Animal model of KW drinking

Male C57BL/6Slac mice at the weight between 18-24 g were housed in the specific pathogen-free facility while orally administered with KW or dH_2O water for 17 days. Mice were inspected daily, and both body weight and water consumption were recorded. On the 17th day, the feces of the mice were collected for determinations of the gut microbiota of the mice.

Gut Microbiome 16S rRNA Gene Sequencing

To elucidate the structural and functional properties of the intestinal microbiota, high-throughput sequencing was employed to analyze the V3-V4 variable region of the 16S ribosomal RNA (rRNA) in the feces: First, genomic DNA from the gut microbiota was extracted using the E.Z.N.A ® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The concentration and purity of the extracted DNA were quantified using a NanoDrop spectrophotometer (ThermoFisher, Waltham, MA, USA). The DNA quality was assessed by

electrophoresis on a 1% agarose gel. Second, the V3–V4 regions of the 16S rRNA gene were amplified with the primer pair 338F (5′-ACTCCTACG GGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by a thermocycler PCR system (GeneAmp 9700, ABI, Foster, CA, United States). After PCR amplification, the product was purified using the AxyPrep DNA Gel Extraction Kit (#AP-GX-250G, Axygen Biosciences, Union City, CA, USA), and the quality of the amplicon was measured with a QuantusTM Fluorometer (Promega, Madison, WI, USA). Amplicon sequencing was conducted on the Illumina MiSeq PE300 system (Promega, San Diego, CA, USA). For the data analysis, raw sequencing data were quality-filtered using fastp (version 0.21.0) and merged by FLASH, followed by clustering into operational taxonomic units (OTUs) at a 97% similarity threshold. The sequences were then aligned against the SILVA138 database for taxonomic assignment.

Statistical Analysis

The R (version 4.1.3) and GraphPad 10 were used to perform general statistical analysis and visualize results via packages vegan (v2.6-4), phyloseq (v1.38.0), tidyverse (v1.3.2), ggpubr (v0.5.0), ComplexHeatmap (v2.10.0) and corrplot (v0.92). Alpha diversity was estimated using the Chao1 and Shannon indices, analyzed by two-tail Mann-Whitney test with a significance threshold set at *P* values less than 0.05. Principal coordinates analysis (PCoA) based on Bray-Curtis matrices with

statistical significance determined by permutational multivariate analysis of variance (PERMANOVA) was conducted to assess the differences in beta diversity between groups. Other data were presented as mean ± SEM and analyzed by two-tail Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

Results

1.KW drinking increased significantly the abundance of several beneficial genera of gut bacterium and decreased significantly the abundance of several harmful genera of gut bacterium of mice

Seventeen days after mice started to drink KW, the KW drinking was found to significantly increase the abundance of several beneficial genera of the gut bacterium of the mice including *Akkermansia*, *Faecalibaculum*, *Ligilactobacillus*, *Lachnospiraceae* and *Roseburia* (**Fig. 1A-1E**). We also found that the KW drinking significantly decreased the abundance of several harmful genera of the gut bacterium including *Clostridioides, Escherichia-Shigella*, and *Enterococcus* (**Figs. 2A-2C**).

2. KW drinking significantly changed the composition of several phyla in the gut microbiota of mice

Seventeen days after mice started to drink KW, the KW drinking was found to change the relative abundance of several phyla in the gut microbiota of mice (**Fig. 3**). While the KW drinking did not affect the abundance of Bacteroidota (**Fig. 4A**), the KW drinking significantly decreased the abundance of Firmicutes (**Fig. 4B**). Notably, the

KW drinking led to a significant decrease in the abundance of Proteobacteria (**Fig. 4C**), while it led to a significantly increase in the abundance of Verrocomicrobiota (**Fig. 4D**).

3. KW drinking significantly increased the diversity and richness of the gut microbiota of the mice

The Venn diagram illustrates that the KW group exhibited a higher number of unique OTUs compared to the Control group (**Fig. 5A**), indicating a notable increase in species richness in the KW group. This finding was further supported by our alpha diversity analysis: The Chao1 index indicated a marked increase in species richness (**Fig. 5B**); and the Shannon index indicated a significant increase in microbial diversity and evenness in the KW group (**Fig. 5C**). Moreover, beta diversity analysis using Principal Coordinates Analysis (PCoA) showed a distinct clustering of microbial communities between the two groups (**Fig. 5D**), suggesting that the KW drinking induced a substantial shift in the composition of the gut microbiota.

Discussion

Our study has obtained the following novel findings: First, KW drinking significantly increased the abundance of several beneficial genera of the gut bacterium of the mice including *Akkermansia*, *Faecalibaculum*, *Ligilactobacillus*, *Lachnospiraceae* and *Roseburia*; second, KW drinking significantly decreased the abundance of several harmful genera of the gut bacterium including *Clostridioides, Escherichia-Shigella* and *Enterococcus*; third, KW drinking significantly increased the

abundance of Verrocomicrobiota of the gut microbiota, while it significantly decreased the abundance of Proteobacteria of the gut microbiota; and fourth, KW drinking significantly increased the diversity and richness of the gut microbiota of the mice.

Our study has shown that the KW drinking led to significant increases in the abundance of several beneficial genera of the gut bacterium, including *Akkermansia muciniphila*, *Faecalibacterium*, *Ligilactobacillus*, *Bacteroides* and *Roseburia.* A number of studies have indicated that *Akkermansia muciniphila* (*A. muciniphila*) is a beneficial gut bacterium that can produce multiple beneficial biological effects, including decreased inflammation, improved glucose metabolism and decreased body fat [11]. There is higher abundance of *A. muciniphila* in the gut of healthy individuals, compared with that of the individuals with metabolic disorders [11]. *Faecalibacterium* is one of the critical types of bacteria in human gut, which has multiple beneficial biological functions such as anti-inflammatory function and production of n-Butyric acid. Both experimental and epidemiological data have indicated the great potential of *Faecalibacterium* for becoming a promising probiotics or live biotherapeutic product [12]: First, there is low abundance of *Faecalibacterium* in the gut of the patients of gastrointestinal diseases, depression and dermatitis; and second, low levels of *Faecalibacterium* are associated with inflammatory conditions. Increasing evidence has also indicated that *Ligilactobacillus murinus*, a member of the *Ligilactobacillus* genus, plays beneficial roles in intestinal metabolism and immune activities of host [13]. There is also a close correlation between the abundance of *Ligilactobacillus murinus* and intestinal health, suggesting its significant potential as a

probiotic [13]. Several species of *Bacteroides* in gut belong to dominant beneficial bacteria, which provide nutrition and vitamins to the host and other intestinal microbial residents by metabolizing polysaccharides and oligosaccharides [14]. The genus *Roseburia* plays beneficial biological roles by metabolizing dietary components which leads to production of butyrate [15]. Butyrate has several important biological functions [16]: Butyrate serves as a link between the intestinal microbiome and epithelium, which leads to generation of fuel of epithelium; butyrate regulates epithelial inflammation through production of anti-inflammatory cytokines; and butyrate in epithelial cells can produce histone modification and altered transcriptional activation which halts cell cycle progression, leading to protective effect against colonic carcinogenesis.

Our study has also shown that KW drinking led to significant decreases in the abundance of three harmful genera of gut bacterium, including *Clostridioides, Escherichia-Shigella*, and *Enterococcus.* *Clostridioides difficile* (*C. difficile*) is the etiological agent for *C. difficile* infection (CDI), which is an antibiotic-associated diarrhea that can be fatal if untreated [17]. *C. difficile* has become an 'Urgent Threat' to U.S. healthcare, since there is an annual CDI burden of approximately 220,000 cases and 13,000 deaths [17]. *Shigella* belongs to harmful gut microbiome, which is causative to bacillary dysentery in humans [18]. Bacillary dysentery is characterized by invasion and inflammatory destruction of the human colonic epithelium [18]. The genus *Enterococcus* is a causative agent of healthcare-associated infections [19]. The majority of enterococcal infections are caused by *Enterococcus*

faecalis and *Enterococcus faecium*, both of which have intrinsic resistance to common antibiotics [19].

Our study has found that KW drinking led to a significantly decreased abundance of Proteobacteria, while it led to significantly increased abundance of Verrocomicrobiota. These observations are consistent with our findings stated above: Since there are multiple genera of harmful gut bacterium in the phylum of Proteobacteria, our finding regarding the KW-produced decrease in the abundance of Proteobacteria is consistent with our findings regarding the KW-produced decreases in the abundance of several genera of harmful gut bacterium; since there are multiple genera of beneficial gut bacterium in the phylum of Verrocomicrobiota, our finding regarding the KW-produced increase in the abundance of Verrocomicrobiota is consistent with our findings regarding the KW-produced increases in the abundance of several genera of beneficial gut bacterium.

Ecological theory predicts that species-rich communities are less susceptible to invasion, suggesting that higher microbial richness and diversity enhance the environmental resilience of gut microbiota. Conversely, low microbial richness and diversity correlate to obesity [20, 21], IBD [22] and *C. difficile*-associated disease [23]. Our study found that KW drinking significantly increases the richness and diversity of the gut microbiota, suggesting that KW drinking can also produce beneficial effects by increasing the richness and diversity of the gut microbiota.

It is of great interest to investigate the mechanisms underlying the effects of KW drinking on the gut microbiota of mice. Our previous cell culture studies and animal

studies have reported that KW has significant antioxidant capacity [8] and anti-inflammatory capacity [9]. Since inflammation and oxidative stress may produce significant effects on gut microbiota [10], it is warranted to test our hypothesis that KW may affect the gut microbiota through its effects on the inflammatory processes and oxidative stress in the gut of mice.

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Figure Legends:

Figure 1. KW drinking increased significantly the abundance of several beneficial genera of the gut bacterium of mice. Seventeen days after mice started to drink KW, the KW drinking was found to significantly increase the abundance of several beneficial genera of the gut bacterium of the mice, including *Akkermansia* **(A)**, *Faecalibaculum* **(B)**, *Ligilactobacillus* **(C)**, *Lachnospiraceae* **(D)**, and *Roseburia* (**E**). Data are presented as mean ± SEM. Statistical significance was determined using two-tail Student's *t*-test. #, $P < 0.05$; ##, $P < 0.01$. N = 6.

Figure 2. KW drinking decreased significantly the abundance of several harmful genera of the gut bacterium of the mice. Seventeen days after mice started to drink KW, the KW drinking was found to significantly decrease the abundance of several harmful genera of the gut bacterium of the mice, including *Clostridioides* **(A)**, *Escherichia-Shigella* **(B)**, and *Enterococcus* (**C**). Data are presented as mean ± SEM. Statistical significance was determined using two-tail Student's *t*-test. ###, $P < 0.001$. N = 6.

Figure 3. KW drinking changed the distribution of several phyla in the gut microbiota of the mice. Seventeen days after mice started to drink KW, relative distribution of several phyla in the gut microbiota of the mice was determined. The dominant phyla include *Bacteroidota* (blue), *Firmicutes* (orange), *Verrucomicrobiota* (green), *Proteobacteria* (red), *Cyanobacteria* (purple), and Others (brown). N = 6.

Figure 4. KW drinking significantly changed the abundance of several phyla in the gut microbiota of the mice. Seventeen days after mice started to drink KW, the abundance of several phyla in the gut microbiota of the mice was determined. While the KW drinking did not affect the abundance of *Bacteroidota* **(A)**, the KW drinking significantly decreased the abundance of *Firmicutes* **(B)**. The KW drinking also significantly decreased the abundance of *Proteobacteria* **(C)**, while it significantly increased the abundance of *Verrucomicrobiota* **(D)** in the gut of the mice. #, $P < 0.05$; ##, $P < 0.01$. N = 6.

Figure 5. KW drinking significantly increased the diversity and richness of the gut microbiota of the mice. (A) The Venn diagram illustrates that the KW group exhibited a higher number of unique OTUs compared to the Con group, indicating a notable increase in species richness in the KW group. **(B)** Alpha diversity analysis on Chao1 index showed a marked increase in species richness. **(C)** Alpha diversity analysis on Shannon index showed a significant increase in microbial diversity and evenness in the KW group. **(D)** Beta diversity analysis using Principal Coordinates Analysis (PCoA) showed a distinct clustering of microbial communities between the two groups, suggesting that KW drinking induced a substantial shift in the composition of the gut microbiota of the mice. **, $P < 0.01$. N = 6.

Fig. 1

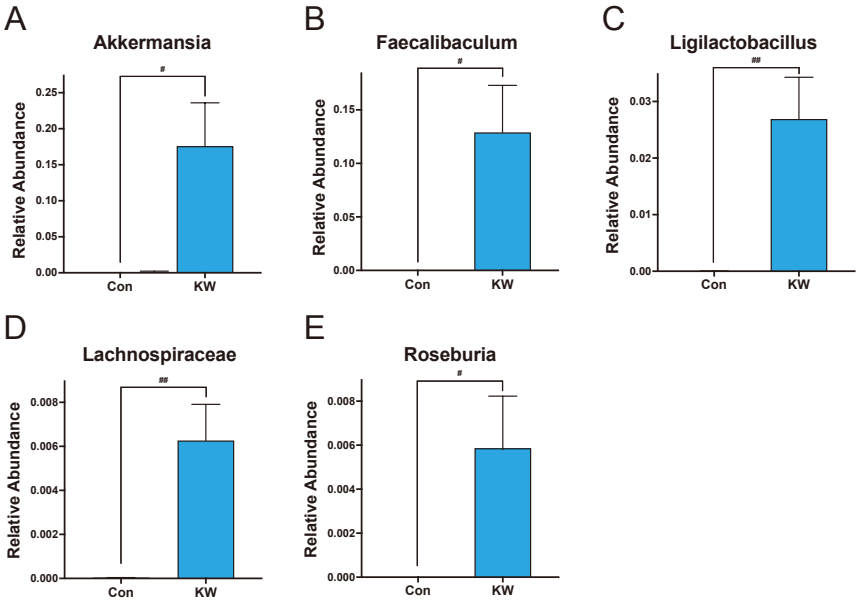
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Fig. 2

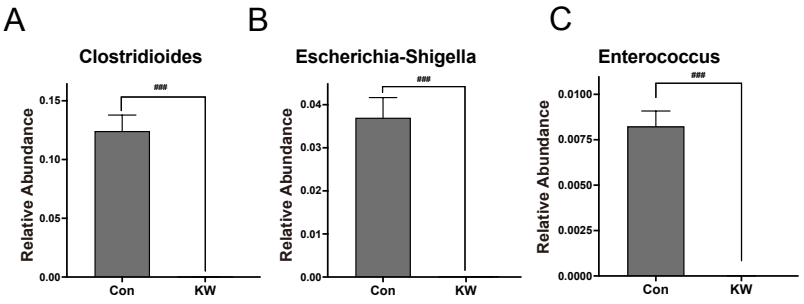
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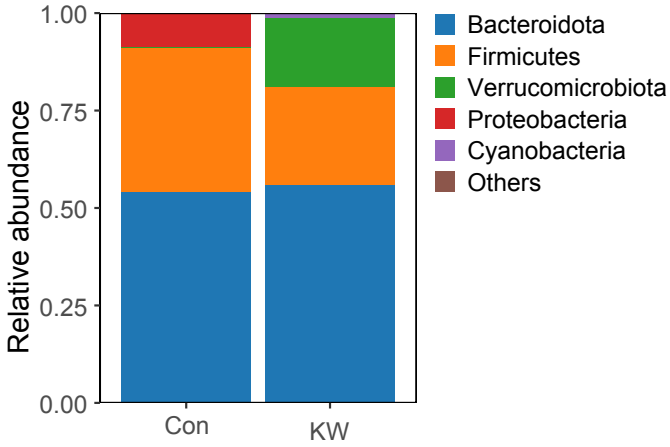
Fig. 3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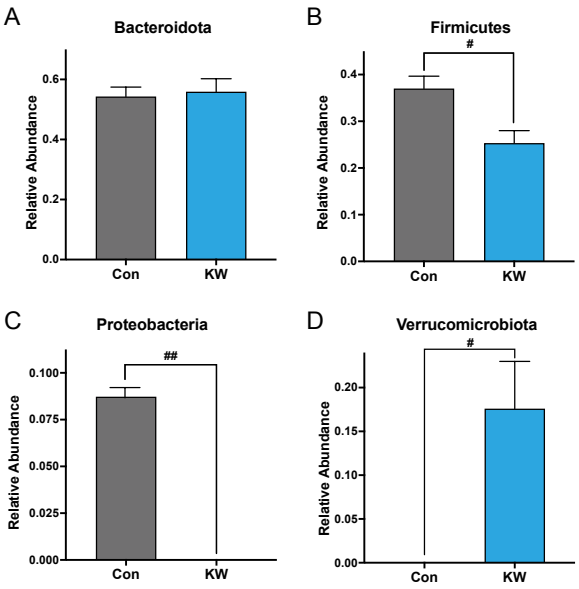
Verrucomicrobiota
Proteobacteria
Cyanobacteria
Others

Fig. 4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Proteobacteria
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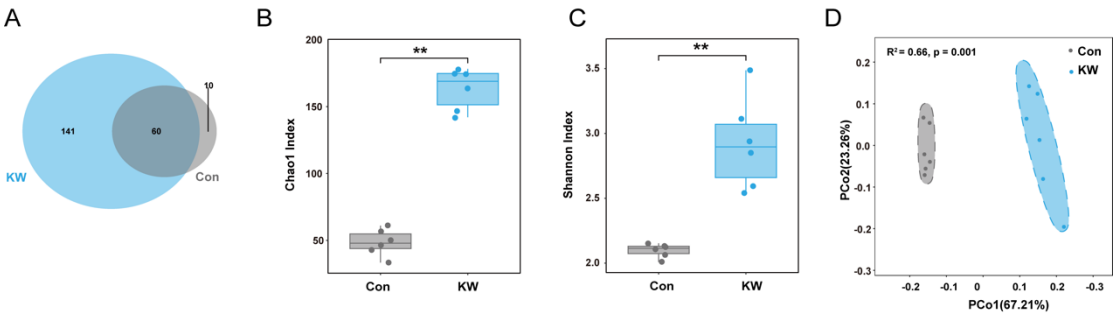
Fig. 5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KW
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150
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Con
KW
C
Shannon Index
3.5
3.0
2.5
2.0
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Con
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D
R² = 0.66, p = 0.001
Con
KW
PCo2(23.26%)
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.2
-0.1
0
0.1
0.2
-0.3
PCo1(67.21%)